Original Article

Characterization and bioprospecting of endophytic Actinomycetota from the leaves of the seagrasses *Thalassia hemprichii* and *Syringodium isoetifolium*

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Abstract

Bacteria of the phylum Actinomycetota are renowned for producing numerous bioactive compounds. Prior research has focused on terrestrial strains, rather than those from marine environments. This study aimed to isolate and characterize endophytic Actinomycetota bacterial strains from the leaves of the seagrasses *Thalassia hemprichii* and *Syringodium isoetifolium* from the coast of Tanzania, to analyze their bioactive metabolites against pathogenic micro-organisms. Fourteen isolates belonging to the genera *Rhodococcus*, *Brachybacterium*, *Microbacterium*, *Kocuria*, *Gordonia*, *Janibacter*, *Flavobacterium*, *Nocardioides*, *Cellulosimicrobium*, *Leifsonia* and *Streptomyces* were identified based on morphological and biochemical analysis, and partial 16S rRNA gene sequences. Among these, six putative novel species are reported. Most of the crude extracts demonstrated antimicrobial activity against at least one of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Candida albicans*. Strains of *Microbacterium* (SID3), *Streptomyces* (THD8), and *Cellulosimicrobium* (SID1) were active against all the tested human pathogens with inhibition zones ranging from 11 to 32 mm. Ten Actinomycetota species exhibited potential for toxic activities. Endophytic Actinomycetota associated with the two studied seagrass species hold significant biotechnological potential for producing biologically active compounds.

Keywords: seagrass species, secondary metabolites, Actinomycetota, cytotoxicity, western Indian Ocean

Introduction

Actinomycetota is a phylum of Gram-positive bacteria characterized by high G+C content in their genomes (Barka *et al*., 2016). Actinomycetota are widely distributed in both terrestrial and aquatic environments and are renowned for their ability to degrade complex polymers such as cellulose, chitin, and lignin (Rodríguez-Fonseca *et al*., 2021) with *Streptomyces* being the type genus, while others are often called rare or non-*Streptomyces* Actinomycetota, as they are less frequently isolated from the environment (Martin-Pozas *et al*., 2023). Additionally, these species have been identified as potential sources of bioactive compounds, with previous studies (Gurushankara *et al*., 2009; Ranjani *et al*., 2016; Fikirini *et al*., 2024) indicating that these microbes are among the richest sources of secondary metabolites. Each strain of Actinomycetota is believed to possess the genetic potential to produce 15–25 secondary metabolites (Gurushankara *et al*., 2009). The production of these antimicrobial agents aids in the biocontrol of fungi and other bacteria. Approximately 10,000 antibiotics have been reported from Actinomycetota, which accounts for 45 % of all bioactive

microbial metabolites discovered (Gurushankara *et al*., 2009). While extensive exploration for new natural products from Actinomycetota strains has been carried out using terrestrial soils (Chaudhary *et al*., 2013; Ngamcharungchit *et al*., 2023), marine Actinomycetota has been less studied. This discrepancy may have limited the discovery of new biologically active molecules from marine environments, as these marine Actinomycetota produce distinct bioactive compounds compared to their terrestrial counterparts (Ngamcharungchit *et al*., 2023). Consequently, it is essential to survey new microbial habitats in the quest for novel bioactive compounds.

Several attempts have been made to isolate and characterize Actinomycetota from the Western Indian Ocean region across both terrestrial and marine environments. For example, Acquah *et al*. (2024) identified genera such as *Streptomyces*, *Actinomadura*, *Actinosynnema*, *Amycolatopsis*, *Gordonia*, *Kribbella*, *Nocardia*, *Nonomuraea*, *Rhodococcus*, *Streptosporangium*, *Saccharopolyspora*, and *Tsukamurella* in South Africa. Similarly, Rado *et al*. (2025) reported 20 Actinomycetota species isolated from marine sponges along the southeast coast of Madagascar. Additionally, Waithaka *et al*. (2017) isolated 16 Actinomycetota strains from the geothermal vents of Menengai Crater in Kenya. In Tanzania, studies by Sosovele *et al*. (2012), Lema *et al*. (2022), Kaale *et al*. (2022), and Fikirini *et al*. (2024) have also isolated and characterized Actinomycetota from various environments. Furthermore, Beepat *et al*. (2016) reported, for the first time, seven sponge-associated Actinomycetota species from Mauritius.

Endophytic Actinomycetota bacteria exist in the internal tissue of plants through symbiotic mechanisms without causing immediate negative effects (Savi *et al*., 2015). They have been isolated from a wide range of plants, including medicinal plants and halophytes like seagrasses. Endophytic Actinomycetota are important microbes and have emerged in recent years as possessing bioactivities against various infections (Rajivgandhi *et al*., 2019). They have been widely studied due to their ability to produce active metabolites. The endophytic relationship of Actinomycetota is also recognized for enhancing plant growth under abiotic and biotic stresses through the production of plant growth regulators, nitrogen fixation, and mineral solubilization (Bouizgarne and Ben, 2014). Common endophytic Actinomycetota include species from the genera *Micrococcus*, *Nocardioides*, *Streptomyces,* and *Rothia* (Masand *et al*., 2015).

Seagrasses are flowering plants inhabiting marine environments, classified as halophytes. They are rooted marine angiosperms capable of completing their life cycle either permanently or partially submerged in water (Aboud and Kannah, 2017). As part of the marine environment, seagrasses are rich in Actinomycetota species occurring as epiphytes or endophytes (Jensen *et al*., 2007). Many of these Actinomycetota remain largely uncharacterized. In contrast, investigations into bacterial communities that live endophytically within seagrass tissues are still rare, with only a few reports available (Jensen *et al*., 2007; Crump and Koch, 2008). In order to identify marine microorganisms capable of producing active metabolites, this study involved the search for endophytic Actinomycetota from seagrass leaves of *T. hemprichii* and *S. isoetifolium* collected from the coastal waters of Mjimwema, Dar es Salaam, Tanzania. These seagrass species were selected based on findings from a previous study on antimicrobial activities against *Salmonella typhi* (Hamisi *et al*., 2023), in which the two seagrass species demonstrated strong antimicrobial properties. Therefore, the aim of this study was to isolate and characterize endophytic Actinomycetota bacteria strains associated with the two seagrasses and to analyze their secondary metabolites against pathogenic microorganisms.

Materials and methods

Sampling

Seagrass leaf samples of *T. hemprichii* (THD) and *S. isoetifolium* (SID) were collected during low tide from Mjimwema (at approximately latitude 06°50'S and longitude 39°21'E), 4 km south of Dar es Salaam harbour. The site is considered unpolluted, with minimal inflows, and shielded from wave action by the Sinda, Kendwa, and Makatumbe Islands (Hamisi *et al*., 2009). The field identification of seagrasses was conducted using the standard guidebook by Oliveira *et al*. (2005). The samples were rinsed with seawater to remove free-floating organisms, placed in sterile polythene bags, and transported in a cool box with ice to the laboratory at the Department of Molecular Biology and Biotechnology, University of Dar es Salaam, for subsequent analysis.

Isolation and identification of endophytic Actinomycetota

Seagrass leaves were surface sterilized following Ramachandrana *et al*. (2019) with minor modifications. Leaves were cut into 6 mm pieces, sterilized in 70 % alcohol for 5 min, treated with 1.5 M sodium

hypochlorite for 5 min, and rinsed with distilled water. A total of 48 leaf fragments were placed on Actinomycete Isolation Agar (AIA) plates supplemented with nalidixic acid (20 µg/ml) and nystatin (50 µg/ml) to inhibit bacterial and fungal growth, respectively. Plates were incubated at 28 °C for seven days. Emerging Actinomycetota were subcultured on fresh AIA plates. To confirm endophytic origin, the final rinse water from surface sterilization was spread on AIA plates and incubated at 28 °C for 48 hours. No growth was observed, indicating successful surface sterilization and the endophytic nature of the isolates.

Morphological characterization involved Gram staining and observation of colony colour, shape, and texture (Lema *et al*., 2022). Biochemical characterization included gelatin, starch, and urea hydrolysis, hydrogen sulfide production, citrate utilization, indole, methyl red, Voges-Proskauer, catalase, and oxidase tests (Chaudhary *et al*., 2013).

Molecular characterization and phylogenetic analysis

The genomic DNA of the endophytic Actinomycetota was extracted using a ZymoBIOMICS DNA Fungal/Bacterial MinPrep kit following the manufacturer's protocol. The quality and quantity were assessed using a Nanodrop One (Thermo Fisher Scientific, USA). PCR amplification of the 16S rRNA gene was conducted as described previously by Stach *et al*. (2003) using Actinomycetota-specific primers (235F-CGCGGCCTATCAGCTTGTTG and 878R-CCGTACTCCCCAGGCGGGG). PCR was performed in 200 µl PCR tubes under the following conditions: initial denaturation at 94 °C for 30 seconds, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 60 °C for 50 seconds, and extension at 68 °C for 1 minute. A final extension step was carried out at 68 °C for 5 minutes, followed by cooling to 4 °C. The PCR products were analyzed on a 1.5 % agarose gel and visualized under a UV transilluminator (Electron, VWR International Ltd.). Sanger sequencing of the PCR products was performed with the same primers (235F' and 878R') using an Applied Biosystems instrument (USA) at Macrogen, Europe (www.macrogen-europe.com). Multiple sequence alignment was conducted using the MUSCLE software packaged in MEGA 11, and phylogenetic trees were constructed using the maximum likelihood method (Tamura *et al*., 2021), with bootstrap values based on 1000 replications using the MEGA 11 software.

Bioprospecting

Test microorganisms

Bacteria *Staphylococcus aureus* (ATCC-25923), *Pseudomonas aeruginosa* (ATCC-27853), *Escherichia coli* (ATCC-35218) and fungi *Candida albicans* (ATCC-14053) were used as the test microorganisms for antimicrobial activity. Prior to use, fresh bacterial cultures were grown on nutrient agar at 37 °C for 24 hours, while fungi were cultured on Sabouraud dextrose agar at 28 °C.

Crude extract

Bioactive compounds from Actinomycetota isolates were extracted via submerged fermentation in 1000 ml Starch Casein Broth, incubated at 28 °C for 30 days at 150 rpm (Zothanpuia *et al*., 2017). Post-fermentation, the medium was centrifuged at 10,000 rpm for 10 minutes, and equal volumes of ethyl acetate were added to the supernatant. The solvent phase was collected, evaporated using a rotary evaporator (BUCHI Labortechnik AG, Switzerland), and the dried residues were dissolved in DMSO and lyophilized for further use.

Determination of the antimicrobial activity

The antimicrobial activity of the crude ethyl acetate extract was evaluated using the agar well diffusion method. Test microorganism suspensions were adjusted to 0.5 McFarland standards (10^8 CFU/ml) and inoculated on Mueller-Hinton Agar for bacteria and Sabouraud dextrose agar for fungi. Wells were bored with sterilized 1000 µl tips, and 100 µl of crude extract (100 mg/ml in DMSO) was added. Plates were incubated at 37 °C for bacteria and 28 °C for fungi for 24 hours. Ciprofloxacin (4 mg/ml) and fluconazole (64 µg/ml) served as positive controls for bacteria and fungi, respectively, while DMSO was the negative control (Remya *et al*., 2023).

Determination of the minimum inhibition concentration

The minimum inhibition concentration (MIC) of the active ethyl acetate crude extract of the isolates against the test microorganisms was determined by the broth microdilution technique using a 96-well microtiter plate (Hamisi *et al*., 2023). The crude extract was two-fold serially diluted from 25 mg/ml to 0.195 mg/ml from a starting concentration of 100 mg/ml. The bacterial pathogens were compared with 0.5 McFarland standards to a final concentration of 1.0×10^8 CFU/ml. Antibiotic (gentamicin sulfate (50 - 0.024 µg/ml)) along with bacterial cultures was used as a positive control, and DMSO containing bacterial cultures was used as a negative control. The plates were incubated at 37 °C

for 24 h. Bacterial growth was indicated by a change in pink colour. The lowest concentration at which no bacterial growth was detected was considered the MIC.

Cytotoxicity test

A brine shrimp lethality test (BST) was carried out to determine the cytotoxic activity of the endophytic Actinomycetota crude extract. The procedures were as described by Hamisi *et al.* (2023), in which stock solutions (40 mg/ml) of all extracts were prepared by dissolving them in DMSO. The volume was then adjusted to 5 ml with artificial sea water prepared by dissolving 3.8 g of sea salt in 1 l of distilled water. Each concentration was tested in duplicate. The negative control contained artificial sea water, brine shrimp, and DMSO (0.6 %) only. The vials were incubated under light for 24 h. The mean percentage mortality was plotted against the logarithm of the concentration using the Microsoft Excel computer program. The percentage mortality was calculated as:

$$\% \text{ Mortality} = \frac{\text{Number of dead brine shrimp larvae} \times 100}{\text{Total number of brine shrimp larvae}}$$

Regression equations obtained from the graphs were used to obtain the LC_{50} values. The toxicity levels of the fractions were determined based on the criteria outlined by Hamidi *et al.* (2014): LC_{50} values above 1000 µg/ml (considered non-toxic), between 500 and 1000 µg/ml (indicating weak toxicity), and below 500 µg/ml (classified as toxic).

Results

Morphological and biochemical characterization of isolated endophytic Actinomycetota strains

Sixty-two strains were isolated from the 48 analyzed seagrass leaf fragments, but only 23 endophytic Actinomycetota strains were used, as the other strains exhibited similar colours. Among the 23 isolates, 65 % (n = 15) were isolated from *T. hemprichii* seagrass, and 35 % (n = 8) were from the seagrass *S. isoetifolium*. A variety of colony colours were observed, including

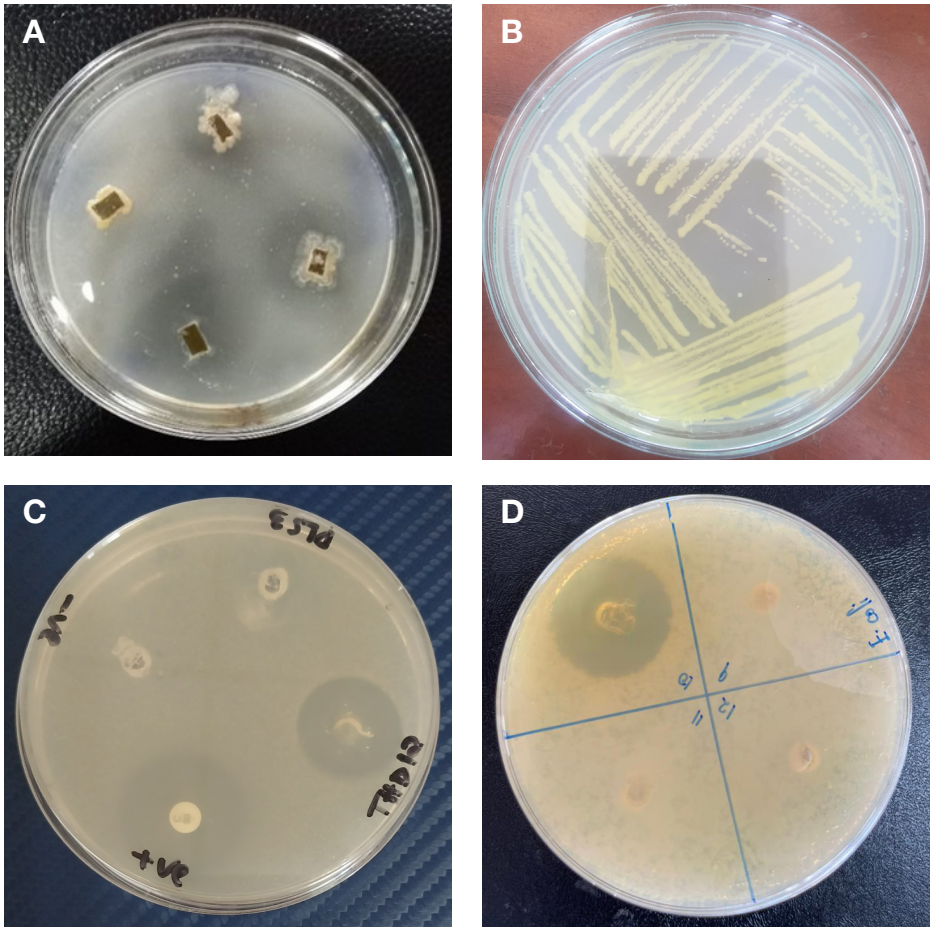


Figure 1. Representative plates of different experiments: (a) shows a mixed colonies emerging from leaf samples; (b) pure culture colonies (pale yellow); (c) inhibition zone shown by two strains (-ve & +ve are negative and positive control respectively); (d) inhibition zone of different strains against *E.coli* (numbers in the plates represent different strains i.e., 9 = THD1, 10= THD5, 11= THD6, 12= THD7.

orange, yellow, pale yellow, brown, and cream (Fig. 1a and b). Morphological features such as colony colour and texture have been employed in the classification of Actinomycetota (Lema *et al*., 2022). Based on these criteria and the references, it was inferred that our isolates belonged to 11 distinct genera of Actinomycetota, as outlined in Table 1. The majority of the isolated species belong to the genera *Kocuria,* followed by *Microbacterium*. Most of the isolates were cocci, while three isolates exhibited a rod shape. Another isolate displayed rod-shaped filaments, while the other isolate was cocci-rod-shaped (Table 1). Additionally, all the Actinomycetota isolates were identified as gram-positive bacteria. The biochemical properties of the isolates (Table 2) showed that they were able to hydrolyze starch, produce catalase, and cannot produce acetyl methyl carbinol from glucose fermentation (Voges-Proskaer test). The majority of the isolates were positive for methyl red, casein, citrate, urea hydrolysis, and gelatin but negative for indole. Other biochemical test results are presented in Table 2.

Molecular analysis

DNA extraction and sequencing were performed on all 23 isolated endophytic Actinomycetota. A comparison of the sequences using the CD-HIT program revealed that some sequences were similar, resulting in the formation of 14 clusters as shown in Table 3, with a representative selected from each cluster. The closest match analysis of the resulting sequences, performed using BLASTn against the GenBank database, revealed that the endophytic isolates from *T. hemprichii* belonged to eight genera (number of strains in brackets) namely, *Rhodococcus* (2), *Brachybacterium* (1), *Microbacterium* (1), *Kocuria* (3), *Gordonia* (1), *Janibacter* (1), *Leifsonia* (1), and *Streptomyces* (1), while the endophytic isolates from *S. isoetifolium* belonged to three genera namely, *Microbacterium* (1)*, Nocardioides* (1), and *Cellulosimicrobacterium* (1). Eight strains were found to have 99–100 % sequence similarity, among which four had 100 % sequence similarity with type strains found in the NCBI database. Six endophytic Actinomycetota strains namely, THD2 (OR939671), THD7 (OR939675), THD11 (OR939667), THD13 (OR939669), THD14

Table 1. Morphological characteristics of endophytic Actinomycetota isolated from *T. hemprichii* (THD) and *S. isoetifolium* (SID).

S/N	Isolates Code	Aerial Mass Color	Reserve Color	Texture	Elevation	Cell shapes	Possible genera	References
1	THD1	Orange	Orange	Rough	Flat	Cocci	*Rhodococcus*	Yuka *et al*., 2012
2	THD2	Cream	Cream	Rough	Flat	Cocci	*Kocuria*	Purty *et al*., 2013
3	THD3	Pale yellow	Pale yellow	Rough	Raised	Cocci	*Brachybacterium*	Gontia *et al*., 2011
4	THD4	Yellow	Yellow	Smooth	Raised	Cocci	*Streptomyces*	Al-Saadi *et al*., 2013
5	THD5	Pale Yellow	Pale yellow	Rough	Flat	Cocci	*Kocuria*	Kovacs *et al*., 1999
6	THD6	Yellow	Yellow	Rough	Flat	Cocci	*Gordonia*	Arenskötter *et al*., 2004
7	THD7	Brown	Brown	Rough	Raised	Cocci- Rod	*Streptomyces*	Al-Saadi *et al*., 2013
8	THD8	Cream	Cream	Smooth	Flat	Cocci	*Janibacter*	Kageyama *et al*., 2007
9	THD9	White	Cream	Smooth	Raised	Cocci	*Streptomyces*	Akshatha *et al*., 2022
10	THD10	Yellow	Yellow	Smooth	Raised	Cocci	*Streptomyces*	Al-Saadi *et al*., 2013
11	THD11	Yellow	Yellow	Smooth	Flat	Cocci	*Rhodococcus*	Dhaouadi *et al*., 2021
12	THD12	Orange	Orange	Smooth	Flat	Rod	*Microbacterium*	Mondani *et al*., 2013
13	THD13	Cream	Cream	Rough	Flat	Rod & Filamentous	*Leifsonia*	Contreras *et al*., 2008
14	THD14	Cream	Cream	Smooth	Flat	Cocci	*Kocuria*	Shashikala *et al*., 2013
15	THD15	Orange	Orange	Smooth	Raised	Cocci	*Microbacterium*	Mondani *et al*., 2013
16	SID1	Pale Yellow	Pale yellow	Rough	Flat	Cocci	*Cellulosimicrobium*	Yoon *et al*., 2007
17	SID2	Yellow	Yellow	Rough	Flat	Cocci	*Cellulosimicrobium*	Yoon *et al*., 2007
18	SID3	Yellow	Yellow	Rough	Flat	Cocci	*Microbacterium*	Laffineur *et al*., 2003
19	SID4	Yellow	Yellow	Smooth	Flat	Cocci	*Cellulosimicrobium*	Yoon *et al*., 2007
20	SID5	Yellow	Yellow	Rough	Raised	Cocci	*Cellulosimicrobium*	Yoon *et al*., 2007
21	SID6	Yellow	Yellow	Smooth	Raised	Rod	*Microbacterium*	Yoon *et al*., 2007
22	SID7	Pale yellow	Yellow	Smooth	Flat	Cocci	*Cellulosimicrobium*	Yoon *et al*., 2007
23	SID8	Yellow	Yellow	Rough	Flat	Rod	*Nocardioides*	Piutti *et al*., 2003

Table 2. Biochemical tests of endophytic Actinomycetota.

S/N	Isolates Code	Oxidase test	Methyl red test	Urea hydrolysis	Indole test	Citrate	Gelatin	Casein
1	THD1	-	+	-	-	+	+	+
2	THD2	-	+	-	-	+	+	-
3	THD3	-	+	-	-	-	+	+
4	THD4	+	+	+	-	+	+	+
5	THD5	+	+	+	-	+	+	+
6	THD6	-	+	+	-	-	+	+
7	THD7	+	+	+	-	-	+	+
8	THD8	+	+	+	-	+	+	+
9	THD9	-	+	+	-	-	+	+
10	THD10	+	+	+	+	-	+	+
11	THD11	-	+	+	-	-	+	-
12	THD12	+	+	+	-	-	-	-
13	THD13	+	+	+	+	+	+	+
14	THD14	+	+	-	-	+	+	+
15	THD15	-	+	+	-	+	+	+
16	SID1	+	-	+	-	+	+	+
17	SID2	+	+	+	-	+	+	+
18	SID3	+	+	+	-	+	+	+
19	SID4	-	+	+	-	+	+	+
20	SID5	+	+	+	-	+	+	+
21	SID6	+	+	+	-	+	+	+
22	SID7	+	+	+	-	+	+	+
23	SID8	+	+	+	-	+	+	+

Table 3. BLAST results for endophytic Actinomycetota with their closest match from the GENBANK database (key: % sequence similarity = percentage sequence, * = putative novel isolate, OR = accession number).

Cluster	Isolate (Accession number)	Size (bp)	Closest match (Accession number)	% identity	Source	Reference
1	THD1 (OR939666)	596	*Rhodococcus* sp. DEE5151 (AY927223)	99.83	Diethyl ether-containing enrichment cultures	Kim and Engesser, 2004
2	THD2 (OR939671)*	577	*Kocuria palustris* (MN629092)	97.75	Brassica seed	Roodi *et al*., 2020
3	THD3 (OR939672)	596	*Brachybacterium* sp.(MG438535)	99.66	Fish	Dong *et al*., 2018
4	THD5 (OR939673)	593	*Kocuria sp*. (MN704221)	99.83	Sponge-associated bacteria	Dat *et al*., 2021
5	THD6 (OR939674)	592	*Gordonia hongkongensis* (NR_152022)	100	Blood culture	Tsang *et al*., 2016
6	THD7 (OR939675)*	580	*Janibacter* sp. (MN703915)	91.60	Sponge-associated bacteria	Dat *et a*l., 2021
7	THD8 (OR939676)	589	*Streptomyces sp*.(MK995563)	100	Weathered rocks of Heshang cave	Wang *et al*., 2022
8	THD11 (OR939667)*	533	*Rhodococcus ruber* (KY174980)	94.44	polycryogel-entrapped	Kuyukina *et al*., 2013
9	THD12 (OR939668)	603	*Microbacterium paludicola* (JQ660113)	99.00	Leaf	Madhaiyan *et al*., 2015
10	THD13 (OR939669)*	586	*Leifsonia psychrotolerans* (NR_108604)	96.63	Moss-covered soil	Ganzert *et al*., 2011
11	THD14 (OR939670)*	578	*Kocuria dechangensis* (NR_137239)	95.50	Saline and alkaline soils	Wang *et al*., 2015
12	SID1(OR939677)	594	*Cellulosimicrobium funkei* (ON678190)	100	Lake	Glekas *et al*., 2022
13	SID3(OR939678)	601	*Microbacterium* sp. (MT533972)	100	Springtail species	Chen *et al*., 2020
14	SID8 (OR939679)*	588	*Nocardioides sp*. 9_72 (HF954526)	98.62	Soil	Siles *et al*., 2014

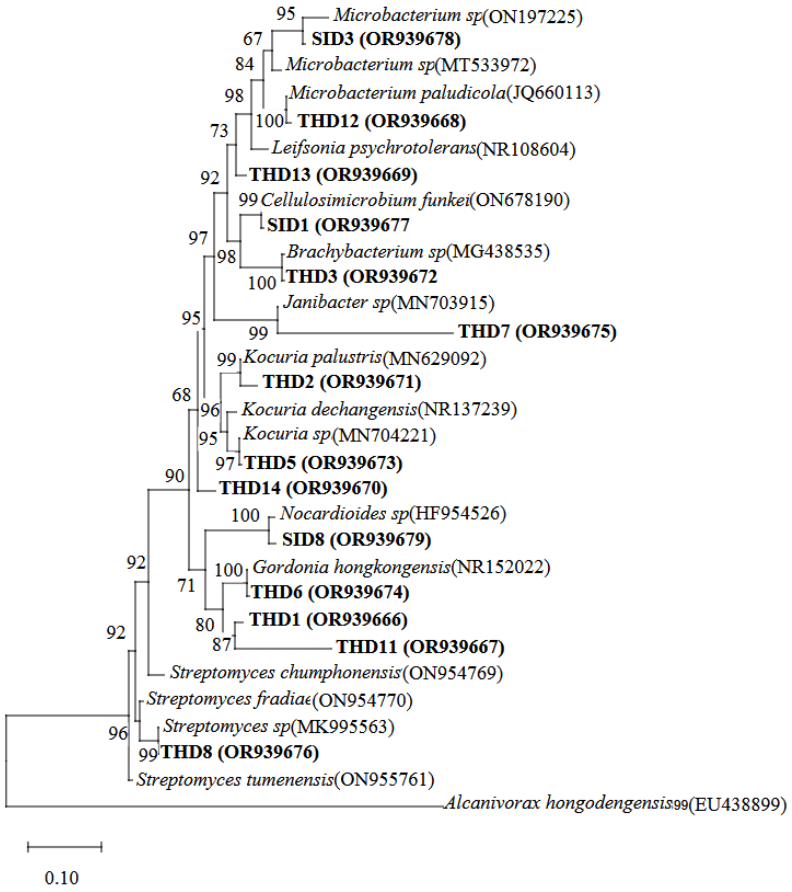


Figure 2. Phylogenetic tree of partial 16S rRNA gene sequences of endophytic Actinomycetota isolates from *T. hemprichii* (THP) and *S. isoetifolium* (SIP) and the type strains of the most closely related genera. A maximum likelihood Heuristic method with a nearest neighbor interchange was used. Bootstrap values are articulated based on 1,000 resamplings of data. Bootstrap values >67 % are shown at branch points. The scale bar indicates 0.1 substitutions per nucleotide position.

(OR939670), and SID8 (OR939679), have 91–98.62 % sequence similarity with the type strains from the Gene Bank. The phylogenetic analysis presented implies considerable diversity of culturable endophytic Actinomycetota from seagrasses, including the isolation of rare Actinomycetota obtained by the maximum likelihood method as shown in Figure 2. The phylogenetic tree shows the relationships and placement of 11 different genera of Actinomycetota species with other type strains. The phylogenetic analysis aligned with BLASTn analysis showed the six putative novel Actinomycetota strains distanced themselves to form their own taxa (Fig. 2).

Antimicrobial and potential of isolates

To verify the production of extracellular antimicrobial metabolites, all 14 isolates were tested against four selected ATCC organisms. The results of antimicrobial activity screening using the agar well diffusion method (Fig. 1c and d) are depicted in Figure 3, showing the diameter of inhibition zones. Twelve isolates (85.71 %) showed antimicrobial activity against at least one of the tested organisms. The majority of the strains (12) showed antibacterial activity against *E. coli* (ATCC-35218), with a maximum zone of inhibition (29 mm) exhibited by *Microbacterium* (THD12) (The names of the strains were derived from the genera identified through molecular characterization, along with their respective laboratory identification numbers). The negative control did not show any antimicrobial activity, while the positive controls exhibited zones of inhibition of 25 mm, 26 mm, 28 mm, and 15 mm on *S. aureus*, *P. aeruginosa*, *C. albicans* and *E. coli*, respectively. Seven isolates (46.67 %) exhibited antibacterial activity against *P. aeruginosa,* with a maximum zone of inhibition of 29 mm by *Cellulosimicrobium* (SID1). On the other hand, 6 isolates (40 %) showed antibacterial activity against *S. aureus*, with the highest zone of inhibition of 32 mm diameter by *Cellulosimicrobium* (SID1). Four (26.67 %) of the isolates exhibited antifungal

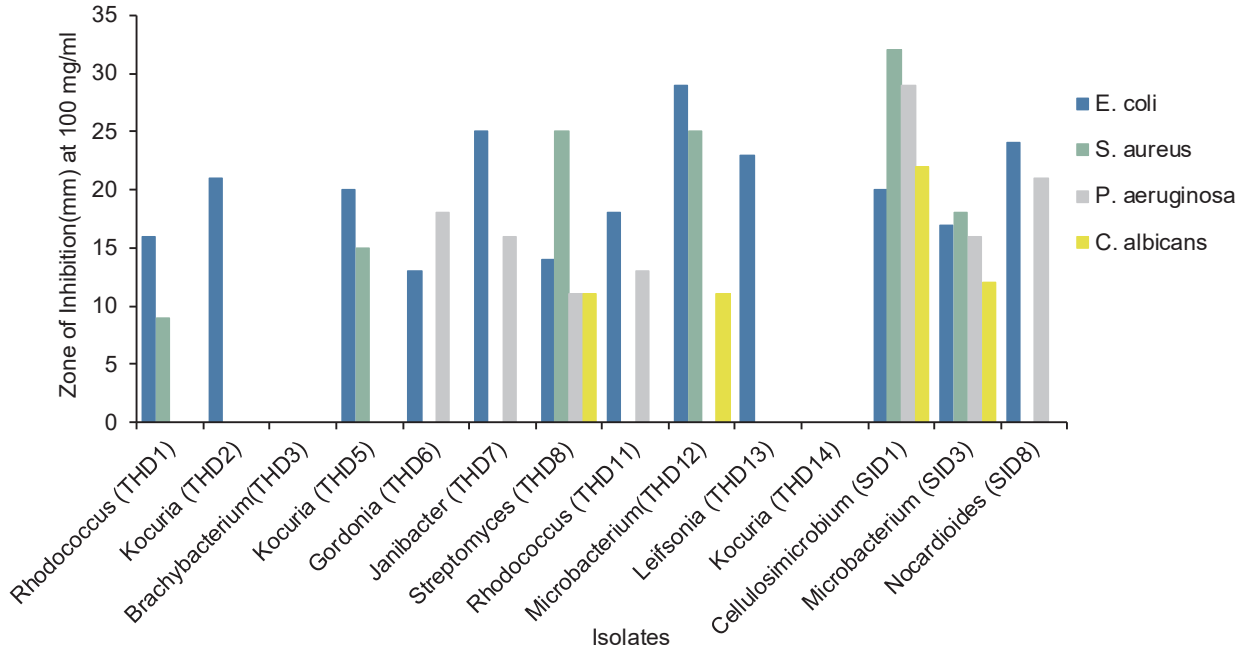


Figure 3. Diameters of the zone of inhibition of the crude extracts of endophytic Actinomycetota isolates.

activity against *C. albicans*, with the largest inhibition zone (22 mm) demonstrated by *Cellulosimicrobium* (SID1). Furthermore, strains *Streptomyces* (THD8), *Microbacterium* (THD12), and *Cellulosimicrobium* (SID1) showed activity against all tested human pathogens.

Minimum inhibition concentration

The microdilution method was used to determine the MIC of the endophytic Actinomycetota strains against all test microorganisms (Fig. 4) at or below the cut-off value of 3.13 mg/ml (Hamisi *et al*., 2023). The *Streptomyces* strain (THD8) exhibited a lower MIC against all tested microorganisms, whereas the lowest MIC was recorded against *C. albicans* (<0.195 mg/ml). Furthermore, the *Microbacterium* strain (THD12) had a lower MIC for three organisms namely, *S. aureus*, *E. coli*, and *C. albicans* (Fig. 4). In addition, *Kocuria* (THD14) had a lower MIC for two organisms, while *Brachybacterium* (THD3) and *Cellulosimicrobium* (SID1) both had lower MICs for only one organism, *S. aureus.*

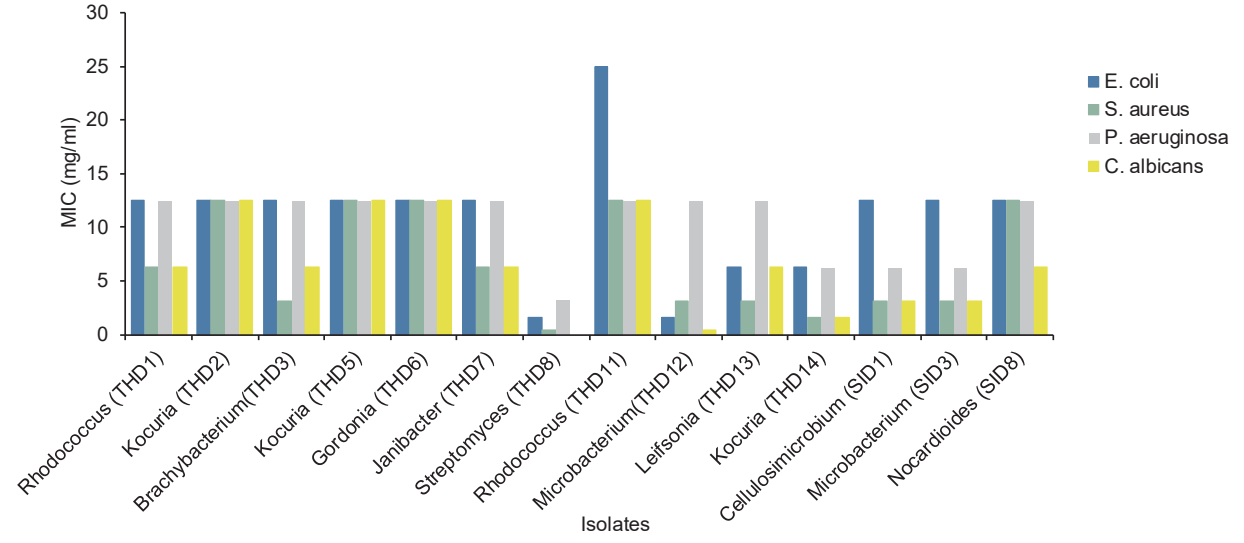


Figure 4. MICs for the marine endophyte Actinomycetota isolates from seagrasses.

Table 4. Brine shrimp activity of endophytic Actinomycetota extracts from *T. hemprichii* and *S. isoetifolium* (The naming of the strains was derived from the genera identified through molecular characterization, along with their respective laboratory identification numbers).

S/N	Isolate extracts	LC50 (µg/ml)	95% Confidence interval (CI)	Regression equation	Regression coefficient(r)
1	*Rhodococcus* (THD1)	1243.4	557.79 -2771.7	y=42.781logx-82.39	0.983
2	*Kocuria* (THD2)	68.423	41.575 -112.61	y=89.298logx-113.88	0.9315
3	*Brachybacterium* (THD3)	67.293	49.398 -91.680	y=73.458logx-84.279	0.9428
4	*Kocuria* (THD5)	728.48	476.97 -1112.6	y=53.923logx-104.35	0.9668
5	*Gordonia* (THD6)	172.81	98.496 -303.20	y=128.34logx-237.17	0.983
6	*Janibacter* (THD7)	81.062	44.833 -146.45	y=99.931logx-140.75	0.9375
7	*Streptomyces* (THD8)	44.383	23.367 -84.297	y=106.75logx-125.84	0.9723
8	*Rhodococcus* (THD11)	141.08	105.41 -188.81	y=78.899logx-119.59	0.978
9	*Microbacterium* (THD12)	50.922	26.247 -98.800	y=109.93logx-137.64	0.9825
10	*Leifsonia* (THD13)	72.361	41.668 -125.67	y=106.98logx-148.93	0.9778
11	*Kocuria* (THD14)	56.125	33.258 -94.710	y=91.93logx-110.8	0.9617
12	*Cellulosimicrobium* (SID1)	379.82	360.51 - 399.12	y=72.628logx-137.35	0.9954
13	*Microbacterium* (SID3)	512.70	430.44 -610.68	y=65.066logx-126.32	0.9535
14	*Nocardioides* (SID8)	3836.7	830.09 -17733.8	y=33.223logx-69.07	1

The brine shrimp lethality assay

Generally, the results showed that most of the endophytic Actinomycetota strains (10) were toxic, with values ranging from 50.922 to 379.82 µg/ml (Table 4). Two strains, *Kocuria* (THD5) and *Microbacterium* (SID3), exhibited weak toxicity. The *Rhodococcus* (THD1) strain from the seagrass *T. hemprichii* was found to be nontoxic, with the lowest LC_{50} of 1243.4 µg/ml, and the *Nocardioides* (SID8) strain from the seagrass *S. isoetifolium* was also found to be nontoxic, with a LC_{50} of 3836.7 µg/ml.

Discussion

Unique bacterial communities associated with seagrass species occupy specific microenvironments around the plant (i.e., epiphytes) and the internal tissues (i.e., endophytes) (Tarquinio *et al*., 2021). The diverse physicochemical conditions found within these microenvironments, including variations in oxygen and nutrient levels, are likely to foster the development of distinct bacterial communities. Seagrass microbiomes play a crucial role in supporting the health and growth of the plants through essential processes such as nutrient supply, including nitrogen fixation associated with seagrass leaves, and detoxification from harmful compounds (Ugarelli *et al*., 2017). In this study, a number of endophytic Actinomycetota species were isolated from the leaves of seagrass *T. hemprichii* and *S. isoetifolium* with *T. hemprichii* having more diversity than *S. isoetifolium*. This difference in number of genera among seagrasses is supported by Martin *et al*. (2018), who indicated that differences exist among the bacterial communities related to the type of seagrass. The results of the present study were also similar to those of Wu *et al*. (2012) who reported that the seagrass *T. hemprichii* harbours a greater number of Actinomycetota genera than other seagrasses. However, Wu *et al*. (2012) isolated Actinomycetota from 10 different genera, but only two of these genera *Streptomyces* and *Microbacterium* matched those isolated in the present study. This difference in composition of the Actinomycetota genera in seagrass *T. hemprichii* can be attributed to the chemical, physical, and biological characteristics of their environment. The endophytic Actinomycetota species identified in this study were similar to the type strains isolated from different sources such as soda lakes, different terrestrial saline and nonsaline soils, leaves, seeds, sponge-associated bacteria, and human clinical specimens, from other studies (Table 3).

Among the isolated 14 endophytic Actinomycetota strains from the two seagrass species, phylogenetic analysis revealed six strains as potential new species: *Kocuria* (THD 2 and THD 14), *Janibacter* (THD7), *Rhodococcus* (THD11), *Leifsonia* (THD13), and *Nocardioides* (SID8). These strains showed less than 98.65 % sequence similarity with their closest matches, a threshold typically used to differentiate between species with higher GC content (Kim *et al*., 2014). This discovery supports the hypothesis that unexplored habitats like marine environments may harbour novel bacterial species (Verna *et al*., 2009). Generally, *Kocuria* spp. have been recovered from various marine habitats, including the seagrass *Cymodocea serrulata* (Jose *et al*., 2014) and *Halodule uninervis* (Bibi *et*

al., 2018); however, these species have not previously been reported in the seagrasses *T. hemprichii* and *S. isoetifolium*. Previous studies show that most species in the genus *Kocuria* are pathogenic and anaerobic causing infections in humans (Ziogou *et al*., 2023). Furthermore, these bacteria are always associated with immunocompromised patients and can lead to more serious infections (Ziogou *et al*., 2023). *Kocuria* has been isolated in various infections, including urinary tract infections, cholecystitis, catheter-associated bacteremia, dacryocystitis, canaliculitis, keratitis, native valve endocarditis, peritonitis, descending necrotizing mediastinitis, brain abscesses, and meningitis (Kandi *et al*., 2016). Setiawan *et al*. (2023) documented the fungal effects of *Kocuria palustris*. The present study, however, explores the antimicrobial properties of newly isolated endophytic *Kocuria* species. Specifically, *Kocuria* (THD2) demonstrated antibacterial activity against *E. coli*, while *Kocuria* (THD14) exhibited both antifungal and antibacterial effects. Additionally, both isolated *Kocuria* strains displayed toxicity, indicating their potential for use in anticancer research (Setiawan *et al*., 2023). The other putative new Actinomycetota species, i.e., *Janibacter*, *Leifsonia,* and *Nocardioides,* have yet to be identified in seagrasses worldwide, while *Rhodococcus* spp. have already been isolated from *Zostera marina* seagrass (Ettinger *et al*., 2021). *Janibacter* species have been reported as causative agents of bacteremia in humans (Sameer and Kunyan, 2005). *Leifsonia* species are known to be enriched in carbon metabolism (Nordstedt *et al*., 2021). *Nocardioides* species are recognized for their ability to utilize a broad spectrum of carbon and nitrogen sources, including unconventional organic compounds and toxic environmental pollutants (Whitman *et al*., 2015). Lee *et al*. (2014) isolated a strain of *Nocardioides* sp. exhibiting biosynthetic potential with the presence of the PKS-II gene, suggesting it could potentially serve as a source of natural products. However, this species has not yet been confirmed to produce any natural products. *Rhodococcus* strains are significant due to their capacity to break down various compounds and generate bioactive steroids, acrylamide, and acrylic acid. They are also involved in the biodesulfurization of fossil fuels (McLeod *et al*., 2006). The present study reveals the antibacterial activities of newly identified *Janibacter* (THD7), *Rhodococcus* (THD11), and *Nocardioides* (SID8) strains against Gram-negative bacteria *E. coli* and *P. aeruginosa,* while *Leifsonia* (THD13) exhibited activity against Gram-negative *E. coli* and Gram-positive *S. aureus*. Furthermore, the *Janibacter* (THD7), *Rhodococcus* (THD11), and *Leifsonia* (THD13) strains were found to be toxic, suggesting their potential as anticancer or toxicity agents, whereas *Nocardioides* (SID8) was non-toxic, indicating its suitability as an antibacterial agent (Yusriadi *et al*., 2019). Further studies are recommended for in vitro and in vivo analyses of the strains to evaluate their efficacy and safety profiles. Additionally, efforts should be made to identify and characterize the bioactive compounds responsible for the observed anticancer and antibacterial effects.

Phylogenetic analysis reveals that the isolated strains *Microbacterium* (SID3), *Streptomyces* (THD8), and *Cellulosimicrobium* (SID1) have a 100 % match with their closest counterparts in the GenBank database. *Microbacterium* spp. have been isolated from various environments, such as soil, insects, human clinical samples, plants, dairy products, and marine environments, including seagrasses such as *T. hemprichii* and *Zostera marina* (Wu *et al*., 2012; Brennan *et al*., 2001). The present report represents the initial identification of *Microbacterium* species within the seagrasses *S. isoetifolium*, and *Microbacterium* spp. are known for their enzymatic production (Li *et al*., 2019), ability to bioremediate, and ability to transport biomolecules such as glucose, fructose, and galactose (Avramov *et al*., 2016). Anteneh *et al*. (2021) showed the antimicrobial activities of species from this genus. Additionally, Graça *et al*. (2023) observed antimicrobial effects in *Microbacterium* strains. Our study emphasizes the robust antimicrobial activity of *Microbacterium* (SID3), which can have applications in pharmaceuticals. The *Microbacterium* strain exhibited toxicity in the brine shrimp test, underscoring the necessity for further exploration of their potential for anticancer (Yusriadi *et al*., 2019). *Streptomyces* is the largest genus within Actinomycetota (Kämpfer 2006) that has been isolated from different terrestrial and marine environments. Some species have been reported from seagrass species such as *T. hemprichii* (Kim *et al*., 2014), *S. isoetifolium* (Ravikumar *et al*., 2012), *C. rotundata,* and *E. acoroides* (Siro and Pipite, 2024). This genus is renowned for its prolific production of natural products exhibiting antifungal, antioxidant, antibacterial, antitumor, and other activities. These compounds have been developed into pharmaceutical products with diverse applications, contributing significantly to the arsenal of antibiotics in current pharmaceutical use (Siddharth *et al*., 2019). *Streptomyces* species are commonly reported as productive sources of secondary metabolites with various biological activities (Martín and Liras, 2022; Lema *et al*., 2022; Fikirini *et al*., 2024). In the present study, the isolate *Streptomyces*

(THD8) showed strong antimicrobial activity against all four pathogenic organisms tested, but this strain was shown to be toxic in brine shrimp tests, indicating its potential antitumor activity (Ettinger *et al*., 2021). *Cellulosimicrobium* has previously been isolated from the seagrass *Thalassia testudinum* (Couto-Rodríguez *et al*., 2009), but is here reported for the first time from the seagrasses *T. hemprichii* and *S. isoetifolium.* Tanveer *et al*. (2021) reported the antioxidant properties of marine *Cellulosimicrobium funkei,* while the present results also suggest antimicrobial activity. The isolated strain of the genus *Cellulosimicrobium* exhibited toxicity, suggesting its potential use in anticancer applications. The finding from this study may also corroborate the previous report by the authors which showed that seagrasses of Tanzania, including the two studied species, had promising antibacterial activities against *S. typhi* (Hamisi *et al*., 2023). The endophytes, including the isolated Actinomycetota strains, may have contributed to the reported antimicrobial activity of seagrasses depicted in various studies. The activities observed in this study call for further studies or analyses on the potential pharmaceutical use of the isolated Actinomycetota species.

Conclusions

In this study, 14 strains of endophytic Actinomycetota from 10 different genera were documented, found in the seagrasses *Thalassia hemprichii* and *Syringodium isoetifolium* at Mjimwema, Dar es Salaam, Tanzania, in the Western Indian Ocean. Notably, six of these strains, namely *Kocuria* (THD2 and THD14), *Janibacter* (THD7), *Rhodococcus* (THD11), *Leifsonia* (THD13), and *Nocardioides* (SID8), are potentially novel species. This study also marks the first documentation of the occurrence of endophytic Actinomycetota species belonging to *Janibacter*, *Nocardioides*, and *Leifsonia* in seagrasses from the Western Indian Ocean. The majority of the isolated Actinomycetota strains belonged to rare Actinomycetota species with antimicrobial capabilities that hold promise for future exploration and utilization. *Microbacterium* (SID3), *Streptomyces* (THD8), and *Cellulosimicrobium* (SID1) strains displayed notably high bacterial inhibitory activity compared to the other studied strains. Furthermore, the toxicity levels of the isolated endophyte Actinomycetota strains revealed varying degrees of toxicity, with some being nontoxic and the majority exhibiting toxicity. These findings suggest the potential of the majority of strains for medicinal applications, especially as anticancer agents. It is recommended that further studies are conducted to corroborate the current finding and identify and characterize the secondary metabolites produced by these isolates, which could provide valuable insights into their pharmacological and industrial potential.

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